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CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 30, 1973

1. Principal Investigator (give title and degrees):
Joseph Mendicino, Ph.D., Associate Professor
Hussein Abou Issa, Ph.D., Senior Research Associate

2. Institution & address.
Department of Biochemistry
University of Georgia
Athens, Georgia 30602 Phone: 404-542-1334

3. Department(s) where research will be done or collaboration provided:
Department of Biochemistry
University of Georgia
Athens, Georgia 30602

4. Short title of study:
The Role of Nicotine, 3', 5'-Cyclic AMP and Related Enzyme Systems in Pulmonary Function and Disease

5. Proposed starting date: June 1, 1973

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The principal aim of the proposed study will be to investigate the characteristics of the enzyme systems in lung and brain which synthesize cyclic adenosine 3', 5'-monophosphate (cyclic 3', 5' AMP) in response to nicotine and its metabolites, and to examine the effects of nicotine and cyclic 3',5' AMP on the multienzyme systems which are, in turn, influenced by these compounds. The specific objectives will be (1) to study the mechanism of action of nicotine on the synthesis of cyclic 3',5' AMP and related enzyme systems in lung and brain, (2) to determine the levels of cyclic 3',5' AMP in control and nicotine treated tissues "in vivo" and in isolated tissues and slices, (3) to determine the activities of adenylcyclase and cyclic 3',5'-AMP phosphodiesterase in control tissues and in tissues treated with nicotine, (4) to examine the effects of nicotine, its metabolites and analogues on hormone responsiveness of control and treated cells derived from lung and brain tissues.

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8. Brief statement of working hypothesis:

See attached

9. Details of experimental design and procedures (append extra pages as necessary)

See attached

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The research will be carried out in the Department of Biochemistry at the University of Georgia. The laboratory is completely equipped with instruments necessary for the studies described in this proposal. Equipment which is available and will be used during this investigation include:

Sorvall refrigerated preparative centrifuges (5), Spinco preparative ultracentrifuges (4), Spinco Model E analytical ultracentrifuge, Zeiss PMQ spectrophotometer, walk-in cold rooms and 30 and 37 incubators adaptable to higher and lower temperatures, Beckman amino acid analyzer, Model 120C (2), paper electrophoresis facilities, beckman pH meters, fraction collectors, waring blendors, homogenizers, facilities for housing of experimental tissues and laboratory animals with well equipped surgical room, tissue culture facilities and equipments. Isotope counting and scanning equipment, including two Packard scintillation counters, Gilford spectrophotometer, model 2000 (2), Gilford spectrophotometer model 240, Beckman DU and DBG, Cary spectrophotometers, model 14 and 15, ultrasonic cell disruptor, lyophilizers and evaporators and the usual equipment for chromatography and other ordinary laboratory procedures. The University has also a general electron microscopy facility having both scanning and transmission microscopes. The procurement of the necessary tissues can be easily arranged. The department has facilities for keeping small laboratory animals. The principal investigator has a good working arrangement with local packing houses, so that large amounts of fresh lung tissue can be obtained from larger animals. Human lung tissue may be obtained at lobectomy from several local hospitals.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Joseph Mendicino	30	-0-
Hussein Abou-Issa	100	REDACTED
Fringe Benefits (14%)		
Technical		
Gerald Hickman	50	REDACTED
Donald McRorie	50	
Fringe Benefits (6%)		
Sub-Total for A		18,450

B. Consumable supplies (by major categories)

Chemicals, glassware	2,000
Isotopes	1,500
Tissues and care of experimental animals	750
Sub Total for B	4,250

C. Other expenses (itemize)

Travel to National Meetings	600
Publication and reference material	900
Sub-Total for C	1,500
Running Total of A + B + C	24,200

D. Permanent equipment (itemize)

RC2B Refrigerated Centrifuge and Heads	3,400
Sub-Total for D	3,400
E. Indirect costs (15% of A+B+C)	3,630
Total request	31,230

15. Estimated future requirements:

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	13,500	4,500	1,500	2,500	2,925	24,925
Year 3	15,000	4,000	1,500	2,500	3,075	26,075

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10035386 10

16. Other sources of financial support.
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Enzyme Interactions in Metabolic Regulation	N.I.H.	$21,805	06/01/72-05/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Biosynthesis of Cell Wall Polysaccharides	N.S.F.	$116,925	06/01/73 to 05/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph Mendicino

Signature Joseph Mendicino Date Jan 29 1973

Telephone 404 (Area Code) 542-1334 (Number) 22 (Extension)

Checks payable to

The University of Georgia

Mailing address for checks

Alan W. Barber
Acting Vice President for Business & Finance
The University of Georgia
Athens, Georgia 30602

Responsible officer of institution

Typed Name Robert C. Anderson

Title Vice President for Research

Signature Robert C. Anderson Date

Telephone 404 (Area Code) 542-3360 (Number) (Extension)

10035386 11

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8. Brief Statement of Working Hypothesis - The exact mechanism by which nicotine exerts its effects on various mammalian tissues is not yet well understood. It is not certain whether it directly increases or decreases the rate of formation of intracellular cyclic 3',5'-AMP under different physiological conditions and thereby controls the rate of specific metabolic processes, or whether the possible secondary effects of nicotine in increasing the levels of norepinephrine, serotonin or dopamine in brain tissues for instance, indirectly stimulates or inhibits the synthesis or breakdown of cyclic 3',5' AMP. Two tissues will be used in preliminary studies, lung tissue, which along with kidney and liver, metabolizes nicotine, and brain tissue which does not degrade this compound. To examine the role of nicotine we propose to utilize a technique for the direct measurement of cyclic 3',5'-AMP levels in slices and various tissues preparations of lung and brain. The possible influence of nicotine on the activities of adenylcyclase and cyclic 3',5' AMP phosphodiesterase in these preparations will be examined. The cyclic 3',5' AMP dependent protein kinases of lung and brain will be isolated and their function in the regulation of specific glycolytic enzymes which change quickly in response to increased levels of nicotine will be evaluated. The working hypothesis of the proposed study is based on the idea that in order to understand the primary effects of nicotine it is necessary to study the properties, interactions and regulation of the enzyme systems which are influenced by the presence of nicotine.

10035386 12

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9. Details of Experimental Design and Procedures.

1. Background

The physiological disposition of C^{14}-labeled nicotine, studied by autoradiographical techniques, showed that nicotine was rapidly distributed throughout the tissues and body fluids. Present evidence indicates that nicotine and/or its metabolites are concentrated in target organs such as the brain, adrenal medulla, lungs, liver and the superior cervical ganglion (1). High concentrations of nicotine accumulate very rapidly in the brain of the guinea pigs after the administration of C^{14}-labeled nicotine (2). Also after intratracheal administration of C^{14}- nicotine, a large amount of radioactivity appears in the lungs (1).

Several studies have been done on the pharmacology of nicotine and smoking as well as the release of catecholamines through the action of nicotine. Silvette et al (3) have shown that the dosage of nicotine derived from smoking was sufficient to cause a release of epinephrine from the adrenal medulla. Westfall and Watts (4) also studied the effects of cigarette smoking on epinephrine secretion in the dog and came to the same conclusion. Several studies (5) have also shown the increase in brain epinephrine following nicotine administration. A great deal of significance was placed on the fact that inhalation of cigarette smoke produced an increase in epinephrine levels of peripheral arterial blood since this is the blood which reaches all tissues (6). Epinephrine and total catecholamine output progressively increase above control levels during periods of heavy smoking and these levels return to normal soon after smoking is stopped (7,8). Several possibilities for the mechanism of amine release by nicotine have been discussed (9). The release could be produced by inhibition of an active storage process or by enhancement of the permeability of the cell membrane making it permeable to various amines in both directions. It has been suggested in several studies (10,11) that smoking in man and nicotine administration in dogs is followed by increased plasma concentrations of free fatty acids. Isotopic studies (12) have shown that the rise is due to an augmented influx of free fatty acids into the circulation. It has been suggested that the increased sympathetic and catecholamine activity associated with the presence of nicotine have a basic role in the free fatty acid response (8,13).

The effect of smoking and nicotine on blood glucose levels was also examined. The results of some studies indicate that nicotine causes a significant increase in blood glucose levels (14,15). The hyperglycemic effect of nicotine and smoking was attributed to an increased epinephrine secretion (16). However, several other studies have shown no significant differences in the blood glucose levels of smokers and non-smokers (17). The molecular action of nicotine and the exact mechanism by which nicotine may affect these biochemical processes is still obscure and clearly needs further study at the molecular level. Several of these processes such as the increased catabolism of carbohydrate as well as the increased lipolysis and release of fatty acids are also elicited by administration of 3',5' cyclic AMP. Cyclic AMP is also thought to be involved in the release of hormones such as insulin, thyroxine, prostaglanidins and anterior pituitary hormones.

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It is therefore possible that nicotine acts directly or indirectly on specific enzyme systems leading to an increased or decreased production of 3',5' cyclic AMP. Cyclic AMP, in turn, might then activates cyclic AMP-dependent protein kinases that in turn stimulate glycogenalysis and thereby raise blood sugar levels. These effectors may also activate a triglyceride lipase at the same time causing increased mobilization of free fatty acids. Increased cyclic AMP levels may also promote the release of epinephrine and histamine in the lungs. An epinephrine-sensitive adenyl cyclase has been identified in the subcellular fractions of the lungs (17) and phosphodiesterase is also present (18). Recently Cyclic AMP has been found to be associated with bronchial asthma (19) and caffeine and epinephrine have been found to act synergistically on increasing cyclic AMP levels in rat lung slices (19). The action of nicotine might be due to a direct effect on the enzyme systems responsible for Cyclic AMP homeostasis in the cell leading to an increased or decreased production of 3',5'-Cyclic AMP, or to an indirect effect through the release of epinephrine. The principal objectives of this study are to determine the site and the molecular action of nicotine by studying the effects of nicotine on the levels of 3',5'-Cyclic AMP as well as on the activities of the enzyme systems responsible for the synthesis and degradation of Cyclic AMP in lungs and brain. An attempt will be made to correlate the relationship between the levels of 3',5'-Cyclic AMP and the activity of the enzymes associated with carbohydrate and lipid catabolism in these tissues.

The coordinated function in living systems requires that the cell maintain homeostasis under varying physiological conditions. These mechanisms must in turn depend on the ability of multienzyme systems to increase or decrease the rates of metabolic processes. An understanding of the factors which influence and regulate these enzyme systems is necessary in order to understand the ability of the tissue or organism to maintain this constantly changing dynamic equilibrium. Nicotine and hormonal factors may exert their regulatory effects by simultaneously stimulating and inactivating specific enzyme reactions involved in the breakdown or synthesis of glucose and glycogen and fat. The ability of various protein kinases and protein phosphatases to alter the activity of key rate limiting enzymes such as fructose 1,6-diphosphatase, glycogen synthetase, phosphofructokinase and phosphorylase will be evaluated. The influence of nicotine on the regulatory enzymes will be being examined to determine which enzyme-enzyme interactions are involved in the regulation of these metabolic pathways.

This system in liver and kidney is capable of responding to stimuli which evoke a response to satisfy extracellular energy requirements and cause stored glycogen or hexose derived from the breakdown of protein to be released as free glucose into the circulatory system. Likewise, the same system must satisfy numerous intracellular energy requirements by degrading glycogen or glucose to pyruvate. Factors which cause a specific biosynthetic process to occur in the cell must also simultaneously stimulate the catabolism of carbohydrate or fat by just enough to satisfy this extra energy requirement. One multienzyme system which often supplies this additional energy requirement is the glycolytic system. The basic question which we will be concerned with in the present studies is how do numerous hormones and nicotine regulate the rate of these multienzyme systems in lung and brain tissue. How is the rate of formation of high energy intermediates by a relatively few catabolic systems coordinated with the rates of the many

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synthetic systems which utilize energy in the cell.

Two 3',5' cyclic AMP-dependent and independent protein kinases have been isolated from kidney extracts in order to study the regulation of glycogen synthetase and phosphorylase in kidney and mammary gland. These two kinases were purified from the soluble and the subcellular membrane fractions of swine kidney, and mammary gland. The soluble kinase is dependent on 3',5' cyclic AMP for activity. Under the conditions employed, the Ka value for cyclic AMP for glycogen synthease I kinase activity was in the range of 60-70 nM. The activation was quite specific for cyclic AMP.

As a working hypothesis in our present studies we assumed that harmones or other agents such as nicotine direct biological processes causing a general increase in catabolic metabolism via 3',5' cyclic AMP in order to satisfy the energy requirements of the specific synthetic processes which they stimulated. The graded response of both catabolism of fat and carbohydrate and the synthetic process to the concentration of hormone has been well documented. It was first thought that their specific effects were mediated through this compound and in some cases this mechanism may apply. As a first approximation we have proposed that physiological changes and other agents which initiate changes in a tissue during cell division, development and other activities have two effects. They trigger a specific signal by means of binding to a specific carrier proteins and phosphorylation of histones turn on a particular synthetic process. They also somewhat non-specifically stimulate adenyl cyclase and increase the rate of formation of 3',5' Cyclic AMP which turns on energy yielding processes such as the catabolic systems for fat and carbohydrate.

It should be noted that the 3',5' cyclic AMP dependent protein kinases which we have isolated from kidney phosphorylate glycogen synthetase, phosphorylase kinase as well as histones and casein. Our present interest in the regulation of glycogen synthetase and phosphorylase in lung and brain arose from the observation that the regulatory proteins also phosphorylate several different enzymes isolated from these tissues when nicotine is administered. Because of these observations we would like to under take a study to examine the enzyme systems in lung and brain which are influenced by increased levels of 3',5' cyclic AMP.

2. SPECIFIC AIMS

The principal aims of this study will be (1) to determine the levels of 3',5' cyclic AMP in lung and brain of animals treated with nicotine and to compare these with control animals. (2) To determine the activities of adenyl cyclase and phosphodiesterase after the administration of nicotine in these tissues (3) to isolate and compare the propertirs and activities of Cyclic AMP dependent protein kinases from the tissues of nicotine treated and control animals. To examine the ability of these "regulatory enzymes" to control the activity of glycolysis and lipolysis by influencing the activity of lipases, phosphorylase, glycogen synthetase and phosphofructokinase in brain and liver. (4) To determine the behavior and responsiveness of these enzyme systems to hormones in the tissues of these animals previously treated with nicotine.

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3. METHODS OF PROCEDURE

Different doses of nicotine ranging from 0.5-1.0 mg/kg of nicotine dissolved in saline will be injected intraperitoneally in rats, or doses ranging from 10-100 ug/kg intravenously, and the animals, together with controls receiving saline, will be sacrificed at various intervals after the administration of nicotine. In case of chronic studies, nicotine at doses ranging from 0.25-1.0 mg/kg will be given subcutaneously 4 times daily for at least 2-3 weeks and then the animals sacrificed. Tissues such as the heart, brain and lungs will be rapidly removed and homogenized in 0.25 M sucrose - 0.05 M Tris - 0.001 M EDTA. Activities of adenyl cylase, phosphodiestrase and protein kinases as well as the levels of 3',5' cyclic AMP in the nicotine-treated animals and controls will be determined by the methods previously developed in this laboratory.

Sensitive enzymatic procedures based on the stimulation of glycogen synthetase kinase activity by 3',5' cyclic AMP have been used to assay the concentration of this compound in tissue extracts. Work in this laboratory has shown that a highly purified protein kinase isolated from kidney is dependent on 3',5' cyclic AMP for activity at the level of 10^{-8}M. This assay procedure measures the decrease in kidney glycogen synthetase activity or the transfer of ^{32}P from ($\lambda^{32}P$) ATP to glycogen synthetase. NaF and theophylline aid in the inhibition of enzymes which degrade the cyclic nucleotide. Controls in which small amounts of 3',5' cyclic AMP were added to the buffer used to prepare the extract showed that none of the nucleotide was lost between the time the tissue was extracted and before it was assayed. Other controls were run in order to ensure that the concentration of the cyclic nucleotide present in the extracts accurately reflected the amount which was present in the tissue. Labeled 3',5' cyclic AMP was added to the homogenizing medium and the nucleotide was reisolated by paper chromatography. The concentration of 3',5' cyclic AMP was then determined by isotope dilution and compared with the radioimmunoassay procedure. The assay procedure developed here has several advantages over existing methods for the measurement of cyclic AMP. The assay can measure levels of cyclic AMP in crude tissue extracts and as low as 10^{-13} moles of cyclic AMP. The method is also specific for cyclic AMP.

Adenylcyclase activity will be determined by incubating the crude extract or fresh membranes at 37° in a reaction mixture containing 40nM Tris-HCl (pH 8.0) 5mM $MgCl_2$, 1 mM cyclic AMP, 8mM phosphoenlpyruvate, 10 ug pyruvate kinase, 1 mM ATP and 0.2 microcuries of ^{3}H-ATP H-ATP (purified on a Dowex column AG50W-X8). At the end of the incubation time, 5 mM cyclic AMP will be added, and the tubes were boiled for 3 minutes. The solution is then passed through a Dowex 50-H^+ column (3 x 65 mm).

The fraction containing cyclic AMP was collected and 0.2 ml $ZnSO_4$ (0.2M) and 0.1 ml of saturated $Ba(OH)_2$ were added. The pH was then adjusted to 7.0 and the supernatant liquid obtained after centrifugation was added to a scintillation fluid and counted.

Cyclic-AMP-Phosphodiesterase activity will be determined by an assay that is based on the following reactions.

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^{3}H-cyclic AMP $\xrightarrow[\text{phosphodiesterase}]{}$ ^{3}H-5-AMP $\xrightarrow[\text{venom}]{\text{snake}}$ ^{3}H-adenosine$_{+Pi}$

The entire reaction could be carried out in a plastic liquid scintillation vial. During a 10 min incubation at 37°, the substrate is converted to labeled ^{3}H-5' AMP by the phosphodiesterase preparation and an excess of snake venom nucleotidase catalyses the conversion of ^{3}H-AMP to tritriated adenosine. The reaction is stopped by the addition of an anion exchange resin that absorbs any unrelated substrate and consequently quenches its radioactivity. The reaction product ^{3}H adenosine is not bound by the resin, thus allowing its radioactivity to be determined after the addition of the scintillation fluid.

Perfusion experiments will be carried out with lung and brain "in situ" by circulating or recirculating medium containing nicotine in the animals. The tissues will be rapidly removed at the end of perfusion and homogenized. The activities of adenyl cyclease, phosphodiesterase, protein kinase as well as the levels of 3', 5' cyclic AMP will be determined.

The effect of nicotine and several of its derivatives on the activity of specific glycolytic and lypolytic enzymes will be examined by adding them to a perfursate at a final concentration of 0.01 mM. Upon completion of the perfusion the lung and brain will be rapidly homogenize and the activity of various glycolytic enzymes, such as phosphorylase, glycogen synthetase phosphofructokinase and pyruvic kinase, will be assayed. The results will be compared with those obtained in control experiments. Some of these enzymes as well as the cyclic 3',5' AMP dependent protein kinases and phosphoprotein phosphatases will be isolated from lung and brain. The effect of nicotine on these isolated enzyme systems will be examined and the results will be compared with data obtained from "in vivo" experiments.

4. SIGNIFICANCE OF RESEARCH

The experiments to be carried out in the course of this study will attempt to establish a more specific mechanism for the mode of action of nicotine and to more clearly define its effects on various mammalian tissues. These studies will also determine the nature of the interaction between 3',5' Cyclic AMP and nicotine. An understanding of the mechanism of action of nicotine either directly or through 3',5' cyclic AMP may help to explain some of the effects which are observed in lung and brain tissues when this compound is present.

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12&13. Biographical sketches of investigator and other professional personnel:

Dr. Joseph F. Mendicino- Birthdate- REDACTED

B.S., Case Inst. of Tech., Cleveland Ohio, Chemistry, 1953.
Ph.D., Western Reserve Univ., Cleveland, Ohio, Biochemistry, 1958.
Postdoctoral, Inst. for Biochem., Invest., Buenos Aires, Biochemistry 1958-1960.
Postdoctoral, Western Reserve Univ., Cleveland, Ohio, Biochemistry 1960-1962.
Associate Professor; University of Georgia, Dept. of Biochemistry, 1968 to present.
Assistant Professor; Ohio State University; Department of Biochemistry, 1962-1968.
Postdoctoral Fellow; Western Reserve University (Dr. Harland Wood), 1960-1962.
Postdoctoral Fellow; Inst. for Biochemical Investigation, Buenos Aires, Argentina (Drs. Luis Leloir) as a Research Fellow of National Foundation, 1958-1960.

The applicant went to the Department of Biochemistry of the School of Medicine at Western Reserve University for graduate training in biochemistry, receiving the Ph.D. degree in biochemistry with a physical chemistry minor in 1958. The graduate work was done with Dr. Merton Utter. Since 1958 he has been continuously engaged in biochemical research. From 1958 to 1960, as a post-doctoral research fellow of the National Foundation, studies were carried out in South America at the Institute for Biochemical Investigation in Buenos Aires, Argentina with Dr. Luis Leloir and from 1960 to 1962 at Western Reserve University with Dr. Harland Wood. The principal investigator is presently an Associate Professor in the Department of Biochemistry at the University of Georgia. A member of the American Society of Biological Chemists and American Chemical Society, Graduate Faculty and Institute of Comparative Medicine, University of Georgia.

Publications

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* Reprints submitted with this proposal

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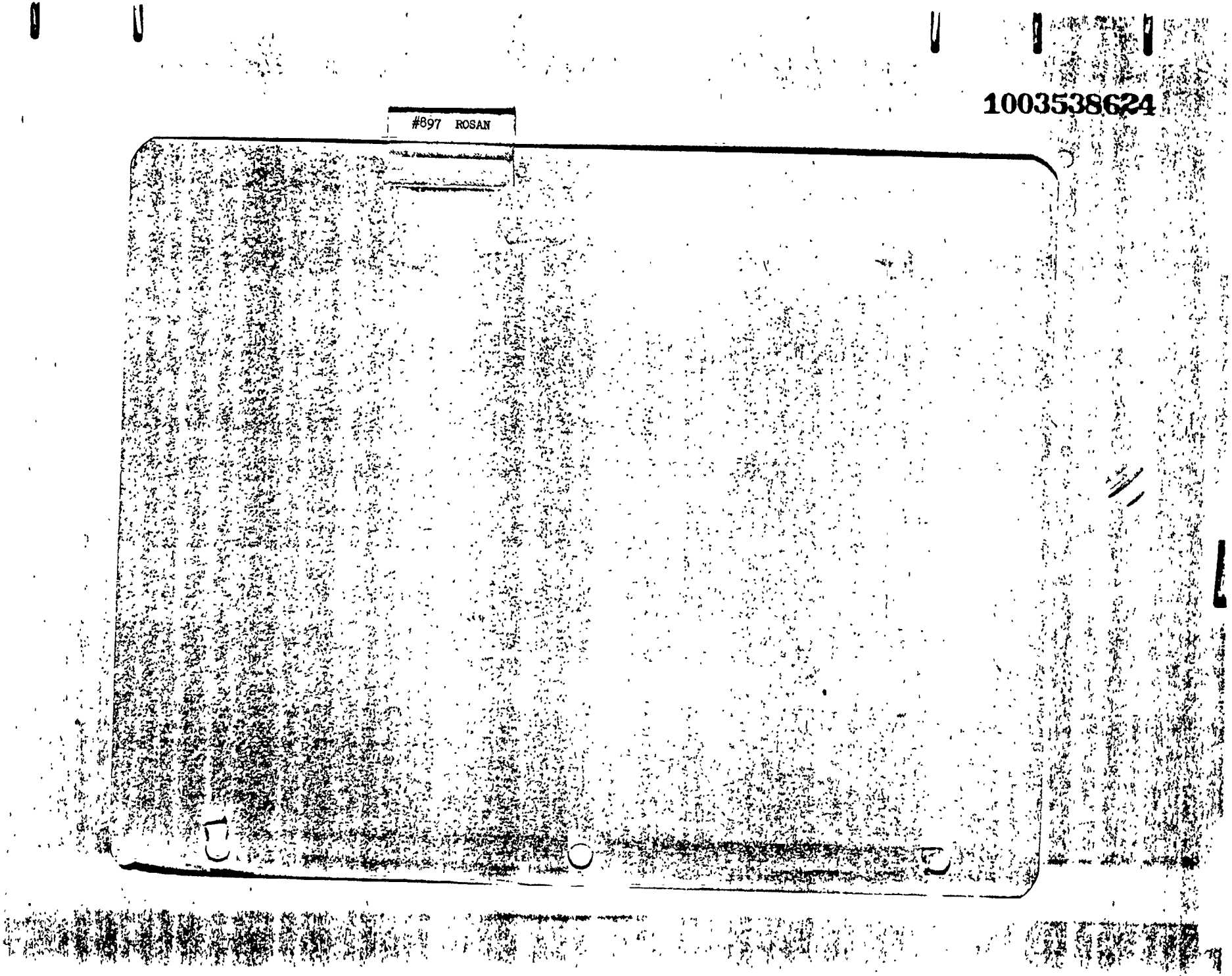

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